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Carbonic anhydrase inhibitors. Characterization and inhibition studies of the most active β-carbonic anhydrase from *Mycobacterium tuberculosis*, Rv3588c

Fabrizio Carta [a], Alfonso Maresca [a], Adrian Suarez Covarrubias [b], Sherry L. Mowbray [c], T. Alwyn Jones [b], Claudiu T. Supuran [a,*]

[a] Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy
[b] Department of Cell and Molecular Biology, Uppsala University, S-751 24 Uppsala, Sweden
[c] Department of Molecular Biology, Swedish University of Agricultural Sciences, SE-751 24 Uppsala, Sweden

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ABSTRACT

The Rv3588c gene product of *Mycobacterium tuberculosis*, a β-carbonic anhydrase (CA, EC 4.2.1.1) denominated here mtCA 2, shows the highest catalytic activity for CO_2 hydration (k_{cat} of 9.8×10^5 s^{-1}, and k_{cat}/K_m of 9.3×10^7 M^{-1} s^{-1}) among the three β-CAs encoded in the genome of this pathogen. A series of sulfonamides/sulfamates was assayed for their interaction with mtCA 2, and some diazenylbenzenesulfonamides were synthesized from sulfanilamide/metanilamide by diazotization followed by coupling with amines or phenols. Several low nanomolar mtCA 2 inhibitors have been detected among which acetazolamide, ethoxzolamide and some 4-diazenylbenzenesulfonamides (K_Is of 9–59 nM). As the Rv3588c gene was shown to be essential to the growth of *M. tuberculosis*, inhibition of this enzyme may be relevant for the design of antituberculosis drugs possessing a novel mechanism of action.

The widely spread human pathogen *Mycobacterium tuberculosis* contains three β-carbonic anhydrase (CA, EC 4.2.1.1) genes in its genome, that is, Rv1284 (encoding for a protein we named mtCA 1), Rv3588c (encoding for mtCA 2) and Rv3273 (encoding for a third enzyme, mtCA 3).[1–3] The catalytic activity and inhibition studies with a range of sulfonamides and one sulfamate of two of these enzymes, that is, mtCA 1 and mtCA 3 have been recently reported,[3–5] whereas Covarrubias et al. reported the X-ray crystal structure of mtCA 1 and mtCA2.[1,2] CAs belonging to the β-class[6] are indeed found in many pathogenic organisms such as fungi (*Candida albicans, Candida glabrata* and *Cryptococcus neoformans* among others)[7–9] and bacteria (*Helicobacter pylori*, *Arthrobacter aurescens*, *Leptospira borgpetersenii*, *Legionella pneumophila* and *Haemophilus influenzae*)[10–14] but they lack from mammals, in which only α-CAs (under the form of 16 different isoforms) are present.[6] Thus, inhibition of such β-CAs started to be considered[3–5,7–12] as a new possible approach for designing anti-infectives (antifungal or antibacterial agents) possessing a different mechanism of action compared to the classical pharmacological agents in clinical use for a long period, for which pathogenic fungi and bacteria developed various degrees of resistance[15,16] The drug resistance problem of antifungals and antibiotics represents a serious medical problem.[17] In this context, *M. tuberculosis* infection is one of the worst example, as multi-drug resistant and extensively multi-drug resistant tuberculosis (TB) is present in many countries.[18] Such drug-resistant mycobacteria show a continuously reduced susceptibility to the clinically used drugs, all of which were developed 30–40 years ago.[19] There is actually a huge interest for novel anti-TB drugs, possessing alternative mechanisms of action compared to the clinically used antibiotics.[19] The complete sequencing of *M. tuberculosis* genome[20] facilitated the identification of possible new drug targets, but more than 60% of this genome encodes proteins whose function is largely unknown at this moment.[1,2,20,21]

In recent contributions from this group[3–5] we have presented the cloning and kinetic characterization of two of the *M. tuberculosis* β-CAs, Rv1284 (mtCA 1),[4] and Rv3273 (mtCA 3),[3] but we have been unable to reclone and express mtCA 2 reported by Covarrubias et al.[1] We also showed that both these enzymes (mtCA 1 and mtC 3) were active CAs, efficiently catalyzing the conversion of CO_2 to bicarbonate and protons, similarly to other α- or β-CAs investigated earlier, which are well-established drug targets.[6–9,22] Furthermore, some clinically used sulfonamides/sulfamates investigated by us as CA inhibitors (CAIs) targeting mammalian α-CAs,[6,23] showed promising in vitro inhibitory

* Corresponding author. Tel.: +39 055 457 3005; fax: +39 055 457 3385.
E-mail address: claudiu.supuran@unifi.it (C.T. Supuran).

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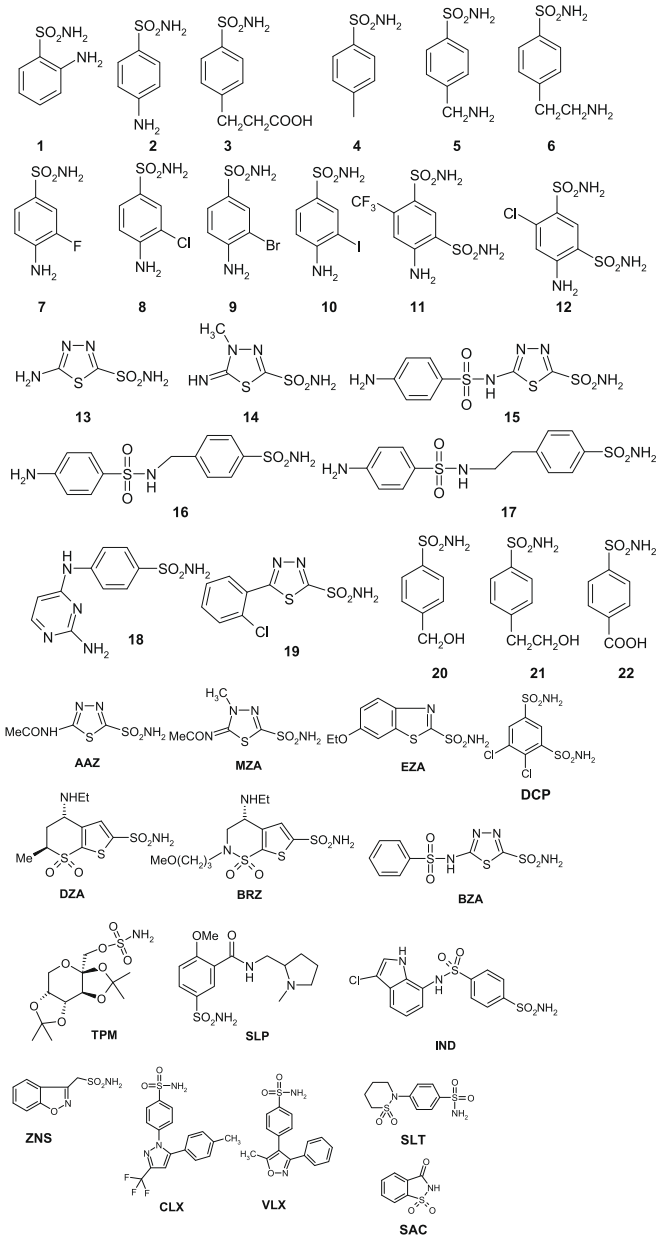
activity against mtCA1 and mtCA 3, with the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the classical antibiotics in clinical use.[3–5] Considering our interest in β-CAs as possible new drug targets, we report here the characterization and inhibition studies with a panel of sulfonamides/sulfamates of the remaining *M. tuberculosis* β-CA, the one encoded by gene Rv3588c and denominated mtCA 2. This enzyme has been reported and characterized crystallographically by Covarrubias et al.[1,2] but its kinetic parameters as well as inhibition has not been investigated for the moment.

We performed a kinetic investigation of purified mtCA 2, prepared as described earlier by one of our groups,[1] comparing its kinetic parameters (k_{cat} and k_{cat}/K_m) with those of thoroughly investigated α-CAs, such as the cytosolic, ubiquitous human isozymes hCA I and II,[6] as well the other two mycobacterial enzymes, mtCA 1 and mtCA 3[3,4] investigated earlier[3,4] (Table 1). As CAs are susceptible to be inhibited by sulfonamides,[3,23,24] data of Table 1 also present the inhibition constant of these enzymes with acetazolamide (**AAZ**), a clinically used drug.[6]

Covarrubias et al.[1] reported that mtCA 2 has catalytic activity as CO_2 hydrase, but no kinetic parameters for this reaction were obtained. Data of Table 1 show that mtCA 2 has the highest catalytic activity for the physiologic reaction among the three mycobacterial enzymes mtCA 1–3, with kinetic parameters in the same range as those for α- or β-CAs investigated earlier, such as hCA I and II.[12,21–23] Indeed, mtCA 2 has a k_{cat} of $9.8 \times 10^5\ s^{-1}$, and k_{cat}/K_m of $9.3 \times 10^7\ M^{-1}\ s^{-1}$, being thus 2.5 times more active than mtCA 1, and 2.3 times more active than mtCA 3 as a catalyst for the physiological reaction. Only the human isoforms hCA II was slightly more active (1.6 times) a catalysts for CO_2 hydration compared to mtCA 2 among the enzymes shown in Table 1, but this is one of the enzymes with the highest turnover numbers known in nature.[6] Furthermore, this catalytic activity of mtCA 2 was highly inhibited by the sulfonamide CAI *par excellence*, acetazolamide **AAZ**, with a K_I of 9.8 nM, much lower than those for the related enzymes mtCA 1 and mtCA 3.[3,4] Furthermore, **AAZ** was a better mtCA 2 inhibitor compared even to hCA II (K_I of 12 nM), and this drug is clinically used to treat glaucoma, a disease due to the imbalanced activity of just this isoform, hCA II, in the eye tissues.[6]

The amino acid sequence of mtCA 2 was aligned with those of the other two ß-CAs present in *M. tuberculosis* (mtCA 1 and mtCA 3)[1–3] and with those of several ß-CAs from other bacteria, such as *Escherichia coli* synT2 (T2) and synT (T), and *Haemophilus influenzae*[13,14] (Fig. 1). As shown by the crystallographic studies of Hogbom's group,[1,2] mtCA 1 and mtCA 2 possess the 3 (or 4) zinc ligands present in the other bacterial β-CAs,[12–14] which are Cys584, His642, Cys645 and Asp586 (Fig. 1, residue numbers based on the *E. coli* CynT2 numbering system).[13] These studies further demonstrated that the residues coordinating the active site Zn(II) ion of mtCA 2 could take on two different structures. In the so-called closed conformation[1] the zinc ion is coordinated by the four protein residues (Fig. 2A) while in the open conformation[2] the aspartyl residue forms a salt bridge with a conserved arginine (Arg584) and is replaced by a water molecule or hydroxide ion (Fig. 2B).[1,2] This is in fact the nucleophile acting on the CO_2 molecule bound to the enzyme, and transforming it to bicarbonate, as in the case of the α-CAs investigated in great detail.[6]

Tables 2 and 3 show mtCA 2 inhibition data with a panel of sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[24] some of which are clinically used drugs,[6] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, sulpiride **SLP**, indisulam **IND**, zonisamide **ZNS**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT** and saccharin **SAC**. The simpler derivatives **1–22** were also included in the study as they represent the most extensively used scaffolds for designing potent or isoform-selective CAIs.[3,25–28] Data for the inhibition of the dominant human isoform hCA II[6] as well as those of the other two *M. tuberculosis* enzymes, mtCA 1 and mtCA 3,[3,4] with these compounds are also included in Table 2, for comparison reasons. The following SAR can be observed from data of Table 2:



(i) A number of the investigated derivatives, such as **1**–**12** and **19**–**22** showed modest mtCA 2 inhibitory activity, with activity in the micromolar range, and inhibition constants of 27.7–45.2 μM. It may be observed that these compounds are either simple 2- or 4-substituted benzenesulfonamides incorporating amino, alkylamino, carboxyalkyl, carboxyl or hydroalkyl moieties (**1–6** and **20–22**), halogeno-substituted sulfanilamides (**7–10**) or benzene-1,3-disulfonamide derivatives (**11** and **12**). Generally, all these compounds were more effective mtCA 1 and mtCA 2 inhibitors (K_Is in the low micromolar or even submicromolar range, Table 2).

(ii) Activity in the low micromolar range has been observed for six of the investigated derivatives of Table 2, that is, **13, 14, 16–18** and **DCP**, with K_Is in the range of 2.01–3.21 μM. These

Table 1
Kinetic parameters for the CO_2 hydration reaction[23] catalyzed by the α-hCA isozymes I, II at 20 °C and pH 7.5 in 10 mM HEPES buffer, and the three *Mycobacterium tuberculosis* enzymes Rv1284 (mtCA 1), Rv3273 (mtCA 3) and Rv3588c (mtCA 2) at 20 °C, pH 8.3 in 20 mM Tris–HCl buffer and 20 mM NaCl and their inhibition data with acetazolamide **AAZ**

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I	Moderate	2.0×10^5	5.0×10^7	250
hCA II	Very high	1.4×10^6	1.5×10^8	12
mtCA 1	Moderate	3.9×10^5	3.7×10^7	480
mtCA 3	Moderate	4.3×10^5	4.0×10^7	104
mtCA 2	High	9.8×10^5	9.3×10^7	9.8

compounds are either heterocyclic derivatives (**13** and **14**, the acetazolamide and methazolamide precursors), sulfanilyl-sulfonamides **16** and **17**, as well as the pyrimidyl-substituted benzenesulfonamide **18**. Dichlorophenamide **DCP** is the only disulfonamide having this interesting and rather effective mtCA 2 inhibitory activity (compared to the structurally related **11** and **12** discussed above, which showed a much weaker inhibitory activity). It may be observed that the elongation of the inhibitor molecule **5** and **6** by means of a sulfanilyl moiety, such as in **16** and **17**, leads to a roughly 10 times increase of the inhibitory power of the corresponding sulfonamide against mtCA 2, which may be an important hint for drug design purposes.

(iii) Submicromolar mtCA 2 inhibitory activity has been observed for a rather large number of derivatives, such as **15**, **MZA** and **BRZ**–**SAC**, which showed K_Is in the range of 127–978 nM (Table 2). Compound **15** is structurally related to **16** and **17** discussed above, but it has the acetazolamide head, whereas most other compounds are heterocyclic sulfonamides in clinical use, except **TPM** which is a sulfamate. These data clearly show that many chemotypes lead to effective, submicromolar mtCA 2 inhibitors. Many of these compounds also effectively inhibit the other two mycobacterial CAs as well as hCA II (Table 2).

(iv) Very effective mtCA 2 inhibitors were acetazolamide **AAZ** (K_I of 9 nM), etoxzolamide **EZA** (K_I of 27 nM) and dorzolamide **DZA** (K_I of 99 nM). These are very encouraging data, as we detected CAIs with an affinity <100 nM for mtCA 2, but on the other hand, all these compounds are very potent inhibitors of most mammalian (host) CA isoforms,[6] which make them less appropriate for developing inhibitors targeting specifically β-CAs. Thus, we decided to prepare compounds which may have better afinity for mtCA 2 but at the same time behave as weaker hCA II inhibitors than the clinically used drugs **AAZ, EZA** or **DZA** discussed above. In the next section we shall present this drug design studies.

In a recent study we observed that several diazenylbenzenesulfonamides act as weak-moderate inhibitors of the ubiquitous, house-keeping human isoforms hCA I and II.[29a] Thus, by using this observation and data reported here, in Table 2, showing that compounds with an elongated molecule such as **15**–**17** possess good (low micromolar) mtCA 2 inhibitory activity, we decided to investigate a series of recently reported[29b] diazenylbenzenesulfonamides **23** and **24**, derived from sulfanilamide or metanilamide.

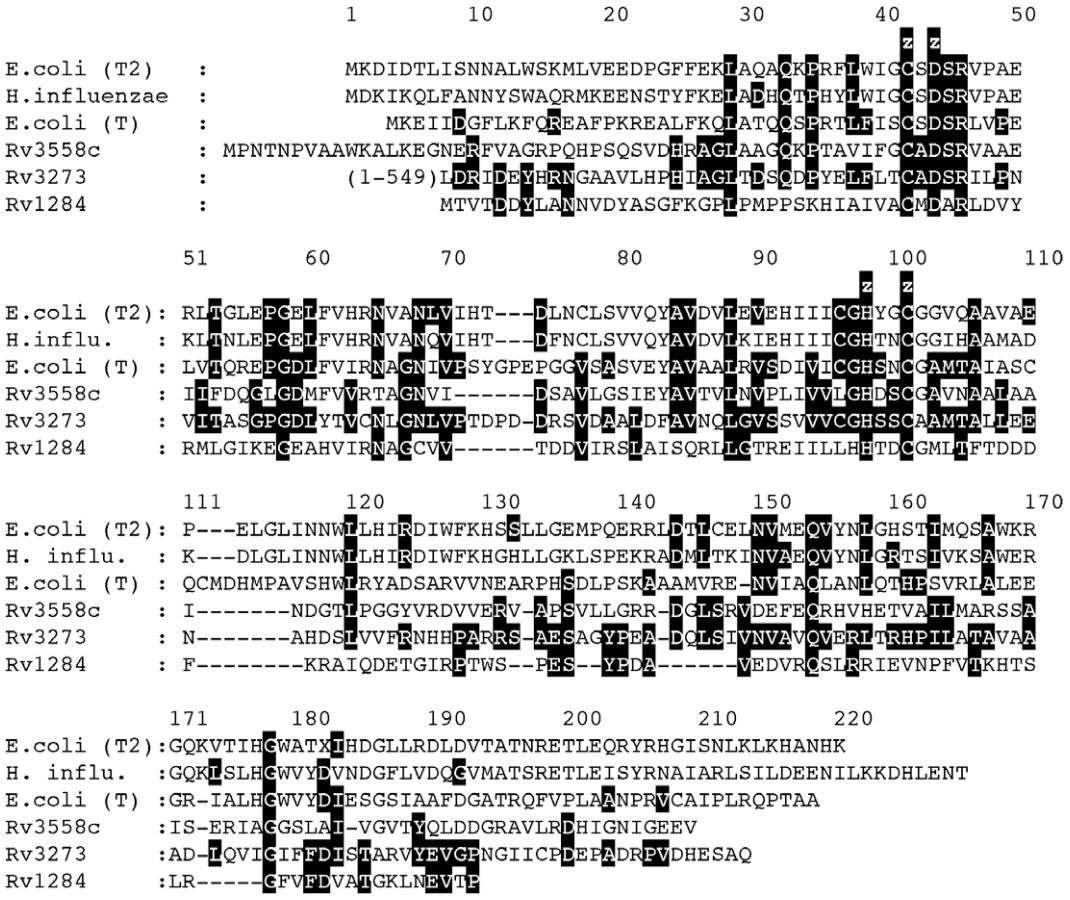


Figure 1. Conserved amino acid residues between the three mycobacterial CAs (Rv3588c, Rv3273 and Rv1284) and other bacterial β-CAs are indicated by a black box. The three/(four) zinc-binding residues, ^{42}Cys, (^{44}Asp), ^{98}His, and ^{101}Cys are indicated by the 'z' sign (residue numbers are based on the *E. coli* CynT2 numbering system).[13]

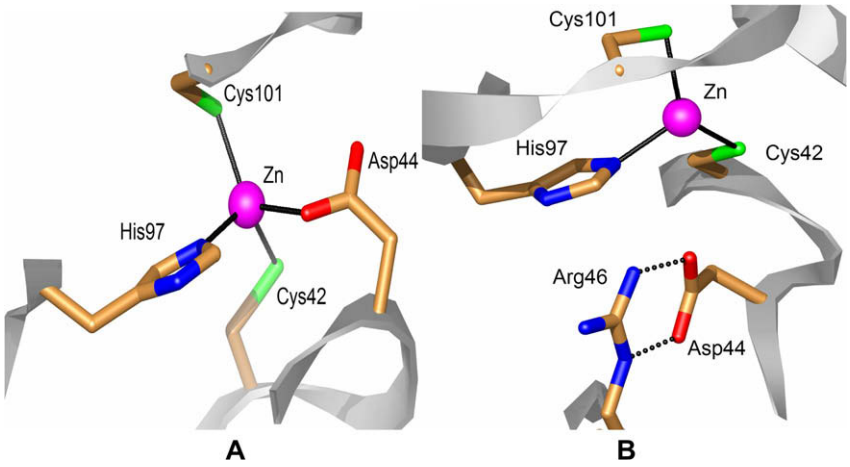


Figure 2. Coordination of the Zn(II) ion in the β-CA encoded by the gene *rv3588c*, that is, mtCA 2. (A) Closed active site, with the Zn(II) ion (violet sphere) coordinated by a histidine, two cysteines and one aspartate residue. (B) Open active site, with three protein ligands coordinated to Zn(II); the aspartate makes a salt bridge with a conserved arginine residue in all β-CAs.[1,2]

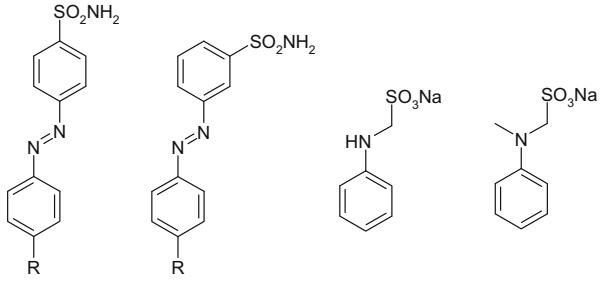
Table 2
Inhibition of hCA II, and *M. tuberculosis* enzymes mtCA 1–3 with sulfonamides **1–22** and 15 clinically used derivatives **AAZ–SAC**

Inhibitor	hCA II[a] (nM)	K_I*		
		mtCA 1[b] (μM)	mtCA 3[c] (μM)	mtCA 2[d] (μM)
1	295	9.23	6.24	33.7
2	240	9.84	7.11	29.6
3	495	7.93	7.83	28.4
4	320	4.92	7.02	38.9
5	170	8.69	7.33	30.7
6	160	9.56	3.42	29.1
7	60	8.74	7.90	28.9
8	110	7.52	1.51	27.7
9	40	0.186	7.32	31.6
10	70	7.71	5.81	32.4
11	63	8.10	2.35	29.6
12	75	1.72	21.7	32.5
13	60	11.54	7.63	2.09
14	19	12.65	7.92	2.38
15	2	0.905	3.10	0.978
16	46	0.612	2.21	3.21
17	50	0.853	0.170	2.29
18	33	0.750	0.091	2.63
19	12	7.48	7.60	45.2
20	80	9.56	7.82	38.3
21	125	5.51	2.51	34.5
22	133	8.21	7.40	39.2
AAZ	12	0.481	0.104	0.009
MZA	14	0.781	0.562	0.66
EZA	8	1.03	0.594	0.027
DCP	38	0.872	0.611	2.01
DZA	9	0.744	0.137	0.099
BRZ	3	0.839	0.201	0.127
BZA	9	0.810	0.338	0.467
TPM	10	0.612	3.02	0.474
SLP	40	2.30	7.92	0.266
IND	15	0.097	7.84	0.717
ZNS	35	28.68	0.208	0.876
CLX	21	10.35	7.76	0.713
VLX	43	12.97	7.81	0.682
SLT	9	5.16	6.72	0.664
SAC	5950	7.96	7.15	0.792

[a] Human recombinant isozyme, stopped-flow CO_2 hydrase assay method, pH 7.5, 20 mM Tris–HCl buffer.[24]
[b,c] Bacterial recombinant enzymes, at 20 °C, pH 8.3 in 20 mM Tris–HCl buffer and 20 mM NaCl, from Refs. 3,4.
Data of isoform II are from Ref. 22 whereas data of mtCA 1 and 3 from Refs. 3,4.
[d] Bacterial recombinant enzyme, at 20 °C, pH 8.3 in 20 mM Tris–HCl buffer and 20 mM NaCl, this work.
* Errors in the range of 5–10% of the shown data, from three different assays.

The chemistry for the preparation of these compounds is non-exceptional and involves diazotization of the aminosulfonamide followed by coupling with phenols or amines.[28,29] We have chosen various R moieties to be present in the molecules of the derivatives **23** and **24** (such as hydroxy, amino, methylamino and dimethylamino, as well as the sulfonato ones from **23e, 23f, 24e, 24f** which may induce enhanced water solubility to these compounds, as sodium salts). The isomers **23** and **24** also differ by the *para*- or *meta*-bulky substituent (with respect to the sulfamoyl moiety). Finally, as sulfonates have not been investigated earlier as possible mtCA inhibitors, we also included in the study the intermediates **25** and **26** which have been prepared for obtaining the azo dyes **23e, 23f, 24e** and **24f** (see experimental protocols). Compounds **23–26** have been assayed as inhibitors of hCA II, and mtCA 3 (Table 3). It may be observed that sulfonamides **23** and **24** are indeed less potent hCA II inhibitors compared to the clinically used derivatives of Table 1. Indeed, their inhibition constants against hCA II are in the range of 88–665 nM (compared to 3–43 nM for the clinically used compounds **AAZ–SLT** of Table 1, which are all very potent, low nanomolar hCA II inhibitors). On the other hand, the two sulfonates **25** and **26** were very weak hCA II inhibitors (K_Is of 58.3–63.6 μM). However, the mtCA 2 data with the new compounds are indeed quite interesting, as the following SAR was observed. First, all the *para*-substituted azo dyes **23** were much more effective mtCA 2 inhibitors compared to the corresponding *meta*-substituted derivatives **24**. Thus, the metanilamide derivatives are less effective than the sulfanilamide ones. For the sulfanilamide derivatives **23**, the dimethylamino-substituted compound was the least effective mtCA 2 inhibitor (K_I of 5.48 μM), whereas the compounds possessing OH, NHMe and NH_2 moieties as substituents to the benzenediazenium system were better inhibitors, with K_Is of 346–955 nM. Thus, a very small structural change in the molecule of these compounds (e.g., an additional methyl moiety in the amino, methylamino or dimethylamino compounds **23b–d**, leads to drastic changes of inhibitory activity). But the best activity has been observed for the aminomethylene sodium sulfonate derivative **23e** and the corresponding N-methylated analogue **23f**, which showed inhibition constants in the low nanomolar range (K_Is of 45–59 nM). The precursors sulfonates **25** and **26** were on the other hand very weak mtCA 2 inhibitors. It is also important to note that these two compounds show some selectivity as mtCA 2 versus hCA II inhibitors, with selectivity ratios for inhibiting the parasite over the host enzyme of 1.8–2.2 (Table 2). Thus, this drug design strategy may be considered a good one (for the *para*-substituted deriv-

Table 3
Inhibition of CAs of human (hCA II) and mycobacterial CAs mtCA 1–3 with sulfonamides **23** and **24**, the sulfonates **25** and **26**, by a stopped-flow CO_2 hydrase assay[24]



23 **24** **25** **26**

No.	R	hCA II[a]	K_I (μM)*		
			mtCA 1[b]	mtCA 3[b]	mtCA 2[c]
23a	OH	0.665	9.27	12.40	0.678
23b	NH_2	0.106	7.20	8.78	0.955
23c	NHMe	0.093	7.69	9.18	0.346
23d	NMe_2	0.638	6.86	30.7	5.48
23e	$NHCH_2SO_3Na$	0.105	6.78	8.90	0.059
23f	$N(Me)CH_2SO_3Na$	0.104	8.71	9.03	0.045
24a	OH	0.106	8.97	9.23	6.48
24b	NH_2	0.088	7.00	8.68	1.98
24d	NMe_2	0.105	7.54	9.36	2.13
24e	$NHCH_2SO_3Na$	0.107	7.51	9.45	6.56
24f	$N(Me)CH_2SO_3Na$	0.109	63	7.4	6.90
25	—	58.3	8.67	8.90	42.9
26	—	63.6	7.86	9.11	54.0

[a] Human recombinant isozyme, stopped-flow CO_2 hydrase assay method, pH 7.5, 20 mM Tris–HCl buffer.[24]
[b] Bacterial recombinant enzymes, at 20 °C, pH 8.3 in 20 mM Tris–HCl buffer and 20 mM NaCl, from Ref. 28.
[c] Bacterial recombinant enzyme, at 20 °C, pH 8.3 in 20 mM Tris–HCl buffer and 20 mM NaCl, this work.
* Errors in the range of 5–10% of the shown data, from three different assays.

atives) in obtaining effective (low nanomolar) and selective mtCA 2 inhibitors. The *meta*-substituted compounds **24a–24f** were less effective mtCA 2 inhibitors, with K_Is of 1.98–6.90 μM (Table 2).

Screening analysis for genes specifically required for the mycobacterial growth showed that Rv3588c is essential for the bacterial growth in vivo.[30,31] In another elegant study, Miltner et al.[32] identified six invasion-related genes of these bacteria. Constitutive expression of the proteins encoded by these genes showed significantly increased ability of the bacterial invasion into HEp-2 and HT-29 intestinal epithelial cells. One of these genes is homologous to *M. tuberculosis* Rv3273, which showed 1.4–1.6 times increase of infected cell number by *Mycobacterium avium* overexpressing its gene product. These findings indicate that CAs encoded by the Rv3588c and Rv3273 genes, play crucial roles in the bacterial infection process, which is however poorly understood at the present time. Thus, inhibition of the three CAs present in this pathogen (mtCA 1, mtCA 2 and mtCA 3) may have relevance for the design of compounds with anti-TB activity possessing a novel mechanism of action, which may be free of the drug resistance problems encountered with the presently used antibiotics.

In conclusion, we report here the kinetic characterization and the first inhibition studies of the third CA from the widespread human pathogen *M. tuberculosis*, mtCA 2, encoded by the gene Rv3588c, which has been shown to be essential for the growth of the organism. mtCA 2, shows the highest catalytic activity for CO_2 hydration (k_{cat} of $9.8 \times 10^5\ s^{-1}$, and k_{cat}/K_m of $9.3 \times 10^7\ M^{-1}\ s^{-1}$) among the three CAs encoded in the genome of this pathogen. A series of sulfonamides/sulfamates was assayed for their interaction with mtCA 2, whereas some diazenylbenzenesulfonamides were newly synthesized from sulfanilamide/metanilamide by diazotization followed by coupling with amines or phenols. Several low nanomolar mtCA 2 inhibitors have been detected among which acetazolamide, ethoxzolamide and some 4-diazenylbenzenesulfonamides (K_Is of 9–59 nM). As this gene is essential for the growth of *M. tuberculosis*, inhibition of this enzyme may be relevant for the design of anti-TB drugs possessing a novel mechanism of action.

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References and notes

1. Suarez Covarrubias, A.; Larsson, A. M.; Hogbom, M.; Lindberg, J.; Bergfors, T.; Bjorkelid, C.; Mowbray, S. L.; Unge, T.; Jones, T. A. *J. Biol. Chem.* **2005**, *280*, 18782.
2. Suarez Covarrubias, A.; Bergfors, T.; Jones, T. A.; Hogbom, M. *J. Biol. Chem.* **2006**, *281*, 4993.
3. Nishimori, I.; Minakuchi, T.; Vullo, D.; Scozzafava, A.; Innocenti, A.; Supuran, C. T. *J. Med. Chem.* **2009**, *52*, 3116.
4. Minakuchi, T.; Nishimori, I.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2009**, *52*, 2226.
5. Guzel, O.; Maresca, A.; Scozzafava, A.; Salman, A.; Balaban, A. T.; Supuran, C. T. *J. Med. Chem.* **2009**, *52*, 4063.
6. Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 168.
7. (a) Innocenti, A.; Mühlschlegel, F. A.; Hall, R. A.; Steegborn, C.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5066; (b) Innocenti, A.; Hall, R. A.; Schlicker, C.; Mühlschlegel, F. A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 2654; (c) Innocenti, A.; Hall, R. A.; Schlicker, C.; Scozzafava, A.; Steegborn, C.; Mühlschlegel, F. A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 4503.
8. Innocenti, A.; Leewattanapasuk, W.; Mühlschlegel, F. A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4802.
9. (a) Schlicker, C.; Hall, R. A.; Vullo, D.; Middelhaufe, S.; Gertz, M.; Supuran, C. T.; Mühlschlegel, F. A.; Steegborn, C. *J. Mol. Biol.* **2009**, *385*, 1207; (b) Innocenti, A.; Winum, J.-Y.; Hall, R. A.; Mühlschlegel, F. A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2642.
10. Nishimori, I.; Minakuchi, T.; Kohsaki, T.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3585.
11. Nishimori, I.; Minakuchi, T.; Morimoto, K.; Sano, S.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 2117.
12. Nishimori, I.; Onishi, S.; Takeuchi, H.; Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 622–630.
13. Cronk, J. D.; Rowlett, R. S.; Zhang, K. Y.; Tu, C.; Endrizzi, J. A.; Lee, J.; Gareiss, P. C.; Preiss, J. R. *Biochemistry* **2006**, *45*, 4351.
14. Smith, K. S.; Jakubzick, C.; Whittam, T. S.; Ferry, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15184.
15. (a) Bennett, J. E.; Izumikawa, K.; Marr, K. A. *Antimicrob. Agents Chemother.* **2004**, *48*, 1773; (b) Fidel, P. L., Jr.; Vazquez, J. A.; Sobel, J. D. *Clin. Microbiol. Rev.* **1999**, *12*, 80; (c) Tsai, H. F.; Krol, A. A.; Sarti, K. E.; Bennett, J. E. *Antimicrob. Agents Chemother.* **2006**, *50*, 1384.
16. Hanage, W. P.; Fraser, C.; Tang, J.; Connor, T. R.; Corander, J. *Science* **2009**, *324*, 1454.
17. Pai, M.; Turpin, R.; Garey, K. *Antimicrob. Agents Chemother.* **2007**, *51*, 35.
18. Dye, C. *Nat. Rev. Microbiol.* **2009**, *7*, 81.
19. (a) Ginsberg, A. M. *Semin. Respir. Crit. Care Med.* **2008**, *29*, 552; (b) Showalter, H. D.; Denny, W. A. *Tuberculosis (Edinb)* **2008**, *88*, S3.
20. Cole, S. T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S. V.; Eiglmeier, K.; Gas, S.; Barry, C. E., III; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M. A.; Rajandream, M. A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J. E.; Taylor, K.; Whitehead, S.; Barrell, B. G. *Nature* **1998**, *393*, 537.
21. Camus, J. C.; Pryor, M. J.; Médigue, C.; Cole, S. T. *Microbiology* **2002**, *148*, 2967.
22. (a) Nishimori, I.; Minakuchi, T.; Onishi, S.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2007**, *50*, 381; (b) Hilvo, M.; Baranauskiene, L.; Salzano, A. M.; Scaloni, A.; Matulis, D.; Innocenti, A.; Scozzafava, A.; Monti, S. M.; Di Fiore, A.; De Simone, G.; Lindfors, M.; Janis, J.; Valjakka, J.; Pastorekova, S.; Pastorek, J.; Kulomaa, M. S.; Nordlund, H. R.; Supuran, C. T.; Parkkila, S. *J. Biol. Chem.* **2008**, *283*, 27799; (c) Vullo, D.; Innocenti, A.; Nishimori, I.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 963.
23. (a) Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 641–648; Supuran, C. T.; Scozzafava, A.; Conway, J. *Carbonic Anhydrase – Its Inhibitors and Activators*; CRC

Press: Boca Raton, New York, London. p 1–363; (c) Stiti, M.; Cecchi, A.; Rami, M.; Abdaoui, M.; Barragan-Montero, V.; Scozzafava, A.; Guari, Y.; Winum, J. Y.; Supuran, C. T. *J. Am. Chem. Soc.* **2008**, *130*, 16130.

24. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5, for α-CAs) or Tris (pH 8.3 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 20 mM NaCl–for β-CAs (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[25] and represent the mean from at least three different determinations. The protein has been prepared and purified as described earlier by Covarrubias et al.[1].
25. Isik, S.; Kockar, F.; Aydin, M.; Arslan, O.; Ozensoy Guler, O.; Innocenti, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 1158.
26. Alterio, V.; Vitale, R. M.; Monti, S. M.; Pedone, C.; Scozzafava, A.; Cecchi, A.; De Simone, G.; Supuran, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 8329.
27. Supuran, C. T.; Clare, B. W. *Eur. J. Med. Chem.* **1999**, *34*, 41.
28. Maresca, A.; Carta, F.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4929.
29. (a) Carta, F.; Pothen, B.; Maresca, A.; Tiwari, M.; Singh, V.; Supuran, C. T. *Chem. Biol. Drug. Des.* **2009**, *74*, 196; (b) Carta, F.; Maresca, A.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 7093.
30. Sassetti, C. M.; Boyd, D. H.; Rubin, E. J. *Mol. Microbiol.* **2003**, *48*, 77.
31. Betts, J. C.; Lukey, P. T.; Robb, L. C.; McAdam, R. A.; Duncan, K. *Mol. Microbiol.* **2002**, *43*, 717.
32. Miltner, E.; Daroogheh, K.; Mehta, P. K.; Cirillo, S. L.; Cirillo, J. D.; Bermudez, L. E. *Infect. Immun.* **2005**, *73*, 4214.